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The DNA Polymerase Gamma R953C Mutant Is Associated with Antiretroviral Therapy-Induced Mitochondrial Toxicity

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We found a heterozygous C2857T mutation (R953C) in polymerase gamma (Pol-γ) in an HIV-infected patient with mitochondrial toxicity. The R953C Pol-γ mutant binding affinity for dCTP is 8-fold less than that of the wild type. The R953C mutant shows a 4-fold decrease in discrimination of analog nucleotides relative to the wild type. R953 is located on the "O-helix" that forms the substrate deoxynucleoside triphosphate (dNTP) binding site; the interactions of R953 with E1056 and Y986 may stabilize the O-helix and affect polymerase activity.

Antiretroviral therapy (ART)-related toxicities predominantly manifest in mitochondrial dysfunction. A critical backbone of ART is nucleoside reverse transcriptase inhibitors (NRTIs). With widespread use of NRTIs, clinical manifestations such as lactic acidosis, lipodystrophy, peripheral neuropathies, cardiomyopathies, and pancytopenia were observed (1–3). These adverse effects of NRTIs were attributed to inhibition of the polymerase gamma enzyme (Pol-γ), responsible for mitochondrial DNA (mtDNA) replication (4). The role of mutant Pol-γ variants in ART-related toxicity has not been systematically investigated. Only two Pol-γ mutations (R964C and E1143G) have been associated with ART-induced mitochondrial toxicity (5, 6).

We hypothesized that Pol-γ mutations might predispose patients toward developing mitochondrial toxicity. We performed a retrospective analysis of data and specimens collected during a prospective, case-control study of ART-induced mitochondrial toxicity (i) to investigate whether Pol-γ mutations are associated with ART-induced mitochondrial toxicity and (ii) to characterize the biochemical effect of these mutations, if any, on Pol-γ activity. The details of the study design have been previously published (7, 8). In brief, the cases comprised HIV-infected individuals identified by their HIV care providers as having symptoms consistent with ART-induced mitochondrial toxicity (2, 9). The study protocol was approved by the Institutional Review Board of the Yale School of Medicine. All participants gave their written informed consent before participation in the study.

The study included 45 African Americans (15 HIV-infected individuals with mitochondrial toxicity [9], cases; 15 HIV-infected individuals without toxicity, positive controls; and 15 HIV-uninfected individuals, negative controls). The demographic and clinical characteristics of participants are illustrated in Table S1 in the supplemental material. We amplified and sequenced the entire polymerase gamma (POLG) genome, comprising 22 exons, of the 45 study participants using 16 pairs of overlapping primers (see Table S2) and a previously described PCR protocol (10). We observed a heterozygous C2857T mutation in exon 18 (see Fig. S1 in the supplemental material) of the POLG catalytic active site, corresponding to a substitution of R953 in the wild type (WT) to cysteine, yielding mutant R953C (Fig. 1A), in one HIV-infected patient with mitochondrial toxicity and observed no mutations in the two control groups. The catalytic site of Pol-γ is highly conserved among species (Fig. 1B), and mutations in this area could lead to depletion of mtDNA and are associated with mitochondrial diseases (6). Therefore, we investigated the mtDNA copy number of the patient with R953C relative those of the two controls. Fragments of the mitochondrial D loop and 18S rRNA nuclear gene were amplified using quantitative reverse transcription-PCR (RT-PCR) and primers (11, 12). The case patient with the R953C mutation had a significantly reduced mtDNA copy number compared with the positive control ($P = 0.001$) and negative control ($P < 0.001$) (Fig. 1C).

Given its location in the catalytic active site, we sought to determine the dissociation constant (K_d) of R953C and WT Pol-γ for the DNA primer-template substrate. Electrophoretic mobility shift assay (EMSA) analysis showed that the R953C Pol-γ binds DNA with an affinity that is not significantly different from that of WT Pol-γ (11.4 ± 0.6 nM and 18.4 ± 0.8 nM, respectively; data not shown) and is consistent with the low nanomolar affinities previously reported for WT Pol-γ (13, 14). To understand the molecular mechanism of the contribution of the Pol-γ R953C mutant to ART-induced toxicity, we employed a pre-steady-state kinetic approach as previously described (13, 14). Since the patient with the Pol-γ R953C mutation had been on lamivudine (3TC) for 10 years prior to diagnosis, we examined incorporation of the natural nucleotide

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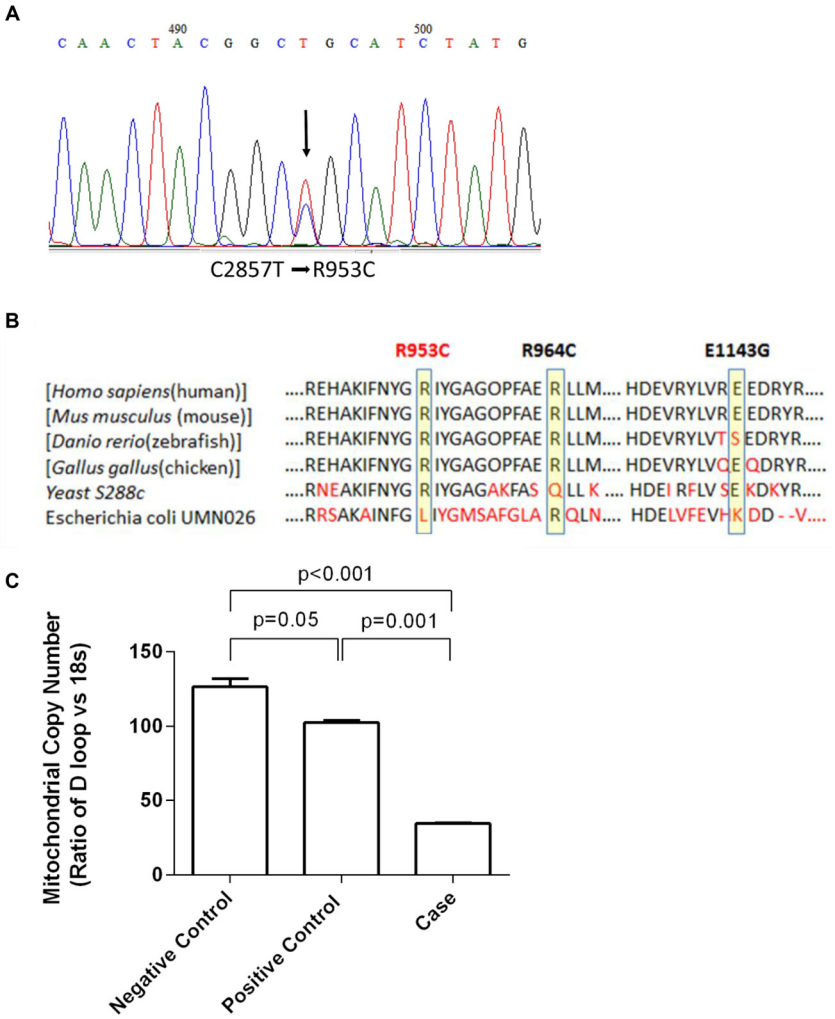

FIG 1 Pol-γ mutation R953C in an HIV-infected individual with hyperlipidemia. (A) Chromatogram showing the sequence around the R953C Pol-γ mutation. (B) Illustration of antiretroviral therapy-associated Pol-γ mutations in conserved domains of Pol-γ. (C) Mitochondrial DNA copy number of R953C patient compared with controls. Fragments of the D loop of the mitochondrial DNA and 18S rRNA nuclear gene were amplified in duplicate in two independent experiments using quantitative RT-PCR. The copy numbers were calculated using serial dilutions of plasmid with known copy numbers of the mtDNA D loop and 18S gene. Data represent averages of the results of two independent experiments.

dCTP relative to the active triphosphate form of 3TC [(-)-3TC-TP]. A series of pre-steady-state burst and single-enzyme-turnover experiments were carried out with WT and mutant R953C Pol-γ holoenzyme, evaluating single-nucleotide incorporation to determine the binding affinity (K_d), maximum incorporation (k_{pol}), and incorporation efficiency (k_{pol}/K_d) for dCTP and (-)-3TC-TP. The R953C Pol-γ K_d for dCTP was 8-fold lower than that of the WT (Table 1), and the k_{pol} for dCTP incorporation by R953C Pol-γ into a growing DNA chain was about 2 times higher than that of the WT, resulting in a 3.6-fold decrease in the efficiency of dCTP incorporation. We also observed a similar reduction in the incorporation efficiency for another natural nucleotide substrate, dTTP (data not shown). On the other hand, the binding affinity of R953C Pol-γ for (-)-3TC-TP was slightly higher than that of the WT whereas the rates of incorporation were the same for the mutant and the WT. This resulted in a 4-fold reduction in the ability of the R953C Pol-γ mutant to discriminate between (-)-3TC-TP and dCTP (Fig. 2A).

To explain the decreased ability of the R953C Pol-γ mutant to discriminate between (-)-3TC-TP and dCTP, we modeled the relationship of deoxynucleoside triphosphate (dNTP) with the side chain of R953C based on the solved crystal structure of the human

TABLE 1 Kinetic parameters describing incorporation of the natural nucleotide dCTP and the NRTI (-)-3TC-TP[a]

Pol-γ variant	Nucleotide	K_d (μM)	k_{pol} (s^{-1})	Efficiency (μM^{-1} s^{-1})	Discrimination ($E_{natural\ dNTP}/E_{analog}$)
Wild type	dCTP	1.3 ± 0.2	67 ± 3	51	5,667
	(-)-3TC-TP	13 ± 5	0.12	0.009	
R953C	dCTP	11 ± 2	150 ± 10	14	1,400
	(-)-3TC-TP	11 ± 5	0.12	0.01	

[a] NRTI, nucleoside reverse transcriptase inhibitor; 3TC, lamivudine; TP, triphosphate; Pol-γ, polymerase gamma; k_{pol}, maximum rate of incorporation; K_d, equilibrium dissociation constant; $E_{natural\ dNTP}/E_{analog}$, efficiency ratio for dCTP/(-)-3TCP-TP.

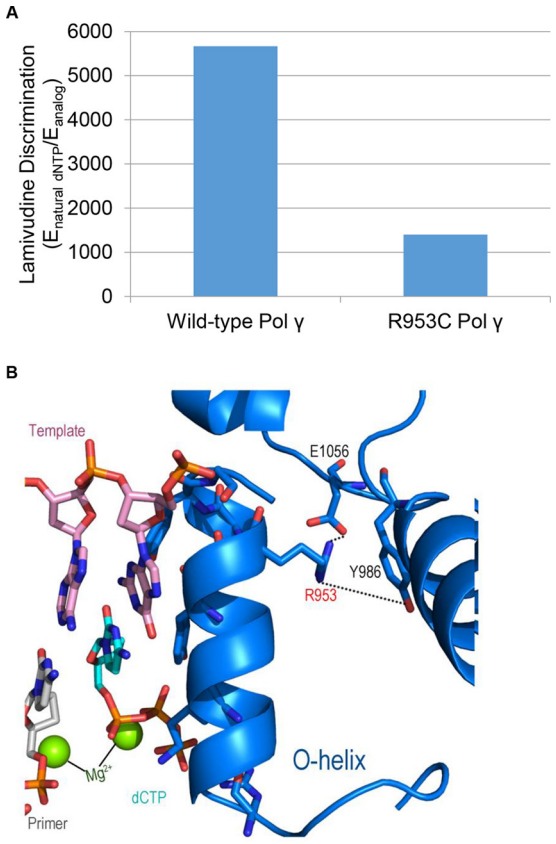

FIG 2 (A) Pol-γ mutation R953C and preference for lamivudine triphosphate. A bar graph illustrates the incorporation efficiency of (-)-3TC-TP in comparison to that of the natural dCTP substrate for the wild type (WT) and R953C of Pol-γ. The Pol-γ R953C mutant demonstrates a 4-fold reduction in discrimination relative to the WT. (B) Pol-γ active site illustrating location of and interaction with R953. R953 is located on the O-helix that forms the substrate dNTP binding site; the interactions of R953 with E1056 and Y986 may stabilize the O-helix and affect polymerase activity. Substitution R953C disrupts the interaction network.

Pol-γ holoenzyme. The catalytic subunit Pol-γA belongs to the Pol I family that structurally resembles a right hand, with domains named the palm, thumb, and fingers. R953 is located on the opposing surface of a positively charged "O-helix" in the fingers domain—the primary binding site for dNTP. The orientation of the O-helix is critically important to DNA synthesis efficiency and fidelity. R953 interacts with E1056 and Y986 (Fig. 2B), two residues that may stabilize the O-helix. Substitution of Arg with Cys abolishes such an interaction and thus could cause slight misalignment of the O-helix, reducing affinity for the incoming nucleotide. (-)-3TC-TP structurally mimics substrate dCTP, but the modification of the ribose moiety to an oxathiolane group might have caused an altered binding mode different from that seen with dCTP; thus, (-)-3TC-TP shows reduced affinity to Pol-γ. This altered binding mode is unaffected by the R953C substitution-induced conformational changes.

R953 is in an evolutionarily conserved (14) region that is critical for normal function of Pol-γ; mutations in this region have been associated with chronic progressive external ophthalmoplegia (PEO) and Alpers' syndrome (15, 16). Indeed, the homozygous Pol-γ R953C mutation has been linked to PEO, while heterozygous family members were asymptomatic (10, 17). Additionally, a molecular analysis of POLG in 2,697 patients identified the R953C mutation in *trans* with the W748S mutation in a patient with moderate depletion of mtDNA levels (61% of the level seen with age-matched controls) (18). Another study of 92 patients with POLG R953C in *trans* with W748S showed the development of mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)-like disorder, an autosomal recessive disorder characterized by severe gastrointestinal dysmotility, cachexia, PEO and/or ptosis, and peripheral neuropathy (19). Functional genetic variants of POLG can be as seen at rates as high as 2% (5), and an aggregate study of 121,421 chromosome samples identified the R953C mutation in 0.001647% (20).

The R964C Pol-γ homozygous mutant was first identified in an HIV-infected individual on NRTI-based ART (3TC and stavudine [d4T]) who developed severe lactic acidosis (5). A detailed pre-steady-state kinetic analysis showed that R964C had a decrease in dTTP incorporation efficiency compared with the wild-type Pol-γ as well as lower d4T-TP discrimination (21), indicating that the R964C Pol-γ mutant predisposes patients to ART-induced mitochondrial toxicity. The second Pol-γ mutation reported to be associated with ART toxicity, E1143G/D, was found in 10 of 69 HIV-infected patients with lipodystrophy (6). However, the Pol-γ E1143G mutant did not affect the activity of the enzyme (22, 23). Taking the data together, our finding is consistent with the concept of predisposition of Pol-γ mutations to ART-induced toxicity.

Our study had several limitations. First, the diagnosis of mitochondrial toxicity was not confirmed with a tissue biopsy specimen; therefore, some of the clinical symptoms of the cases could have been misclassified. Second, the small sample size limits the generalization of our finding. The reported prevalence of the R953C mutation is 0.001647% (20); thus, our finding is intriguing and warrants longitudinal (e.g., pre- and post-ART) studies to validate our observations. The strengths of our study were that we had epidemiologic, biochemistry, and mitochondrial biogenesis data in support of our finding.

In conclusion, we report a novel association of Pol-γ R953C mutation with ART-induced toxicity. The Pol-γ mutation was associated with decreased enzyme activity and mtDNA content. On the basis of our finding and previously published data, we hypothesize that Pol-γ mutations and/or polymorphisms might predispose patients to ART-induced mitochondrial toxicity. Studies to investigate Pol-γ mutations that could predispose patients to ART toxicity might inform the selection of appropriate patient-specific NRTIs to avoid ART-induced toxicity.

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REFERENCES

1. **Brinkman K, ter Hofstede HJ, Burger DM, Smeitink JA, Koopmans PP.** 1998. Adverse effects of reverse transcriptase inhibitors: mitochondrial toxicity as common pathway. AIDS **12:**1735–1744. http://dx.doi.org/10.1097/00002030-199814000-00004.
2. **Moyle G.** 2000. Clinical manifestations and management of antiretroviral nucleoside analog-related mitochondrial toxicity. Clin Ther **22:**911–936; discussion 898. http://dx.doi.org/10.1016/S0149-2918(00)80064-8.
3. **Moyle GJ, Datta D, Mandalia S, Morlese J, Asboe D, Gazzard BG.** 2002. Hyperlactataemia and lactic acidosis during antiretroviral therapy: relevance, reproducibility and possible risk factors. AIDS **16:**1341–1349. http://dx.doi.org/10.1097/00002030-200207050-00005.
4. **Lewis W, Dalakas MC.** 1995. Mitochondrial toxicity of antiviral drugs. Nat Med **1:**417–422. http://dx.doi.org/10.1038/nm0595-417.
5. **Yamanaka H, Gatanaga H, Kosalaraksa P, Matsuoka-Aizawa S, Takahashi T, Kimura S, Oka S.** 2007. Novel mutation of human DNA polymerase gamma associated with mitochondrial toxicity induced by anti-HIV treatment. J Infect Dis **195:**1419–1425. http://dx.doi.org/10.1086/513872.
6. **Chiappini F, Teicher E, Saffroy R, Debuire B, Vittecoq D, Lemoine A.** 2009. Relationship between polymerase gamma (POLG) polymorphisms and antiretroviral therapy-induced lipodystrophy in HIV-1 infected patients: a case-control study. Curr HIV Res **7:**244–253. http://dx.doi.org/10.2174/157016209787581409.
7. **Li M, Foli Y, Amakye NY, Klein T, Selvaraj S, Lu L, Paintsil E.** 2015. Antiretroviral therapy-induced toxicity is associated with increased mRNA expression of telomerase. J Clin Pharmacol **55:**1119–1124. http://dx.doi.org/10.1002/jcph.520.
8. **Selvaraj S, Ghebremichael M, Li M, Foli Y, Langs-Barlow A, Ogbuagu A, Barakat L, Tubridy E, Edifor R, Lam W, Cheng YC, Paintsil E.** 2014. Antiretroviral therapy-induced mitochondrial toxicity: potential mechanisms beyond polymerase-gamma inhibition. Clin Pharmacol Ther **96:**110–120. http://dx.doi.org/10.1038/clpt.2014.64.
9. **Kakuda TN.** 2000. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. Clin Ther **22:**685–708. http://dx.doi.org/10.1016/S0149-2918(00)90004-3.
10. **Gurgel-Giannetti J, Camargos ST, Cardoso F, Hirano M, DiMauro S.** 2012. POLG1 Arg953Cys mutation: expanded phenotype and recessive inheritance in a Brazilian family. Muscle Nerve **45:**453–454. http://dx.doi.org/10.1002/mus.22330.
11. **McComsey GA, Kang M, Ross AC, Lebrecht D, Livingston E, Melvin A, Hitti J, Cohn SE, Walker UA.** 2008. Increased mtDNA levels without change in mitochondrial enzymes in peripheral blood mononuclear cells of infants born to HIV-infected mothers on antiretroviral therapy. HIV Clin Trials **9:**126–136. http://dx.doi.org/10.1310/hct0902-126.
12. **Weglewska A, Jakobkiewicz-Banecka J, Wegrzyn G.** 2005. A modified procedure for quantitative analysis of mtDNA, detecting mtDNA depletion. J Appl Genet **46:**423–428.
13. **Sohl CD, Kasiviswanathan R, Copeland WC, Anderson KS.** 2013. Mutations in human DNA polymerase gamma confer unique mechanisms of catalytic deficiency that mirror the disease severity in mitochondrial disorder patients. Hum Mol Genet **22:**1074–1085. http://dx.doi.org/10.1093/hmg/dds509.
14. **Lim SE, Ponamarev MV, Longley MJ, Copeland WC.** 2003. Structural determinants in human DNA polymerase gamma account for mitochondrial toxicity from nucleoside analogs. J Mol Biol **329:**45–57. http://dx.doi.org/10.1016/S0022-2836(03)00405-4.
15. **Longley MJ, Graziewicz MA, Bienstock RJ, Copeland WC.** 2005. Consequences of mutations in human DNA polymerase gamma. Gene **354:**125–131. http://dx.doi.org/10.1016/j.gene.2005.03.029.
16. **Kaguni LS.** 2004. DNA polymerase gamma, the mitochondrial replicase. Annu Rev Biochem **73:**293–320. http://dx.doi.org/10.1146/annurev.biochem.72.121801.161455.
17. **Luoma P, Melberg A, Rinne JO, Kaukonen JA, Nupponen NN, Chalmers RM, Oldfors A, Rautakorpi I, Peltonen L, Majamaa K, Somer H, Suomalainen A.** 2004. Parkinsonism, premature menopause, and mitochondrial DNA polymerase gamma mutations: clinical and molecular genetic study. Lancet **364:**875–882. http://dx.doi.org/10.1016/S0140-6736(04)16983-3.
18. **Tang S, Wang J, Lee NC, Milone M, Halberg MC, Schmitt ES, Craigen WJ, Zhang W, Wong LJ.** 2011. Mitochondrial DNA polymerase gamma mutations: an ever expanding molecular and clinical spectrum. J Med Genet **48:**669–681. http://dx.doi.org/10.1136/jmedgenet-2011-100222.
19. **Tang S, Dimberg EL, Milone M, Wong LJ.** 2012. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)-like phenotype: an expanded clinical spectrum of POLG1 mutations. J Neurol **259:**862–868. http://dx.doi.org/10.1007/s00415-011-6268-6.
20. **NCBI.** 2016. dbSNP short genetic variations. National Institutes of Health, Bethesda, MD. http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=11546842. Accessed 10 January 2016.
21. **Bailey CM, Kasiviswanathan R, Copeland WC, Anderson KS.** 2009. R964C mutation of DNA polymerase gamma imparts increased stavudine toxicity by decreasing nucleoside analog discrimination and impairing polymerase activity. Antimicrob Agents Chemother **53:**2610–2612. http://dx.doi.org/10.1128/AAC.01659-08.
22. **Ferrari G, Lamantea E, Donati A, Filosto M, Briem E, Carrara F, Parini R, Simonati A, Santer R, Zeviani M.** 2005. Infantile hepatocerebral syndromes associated with mutations in the mitochondrial DNA polymerase-γA. Brain **128:**723–731. http://dx.doi.org/10.1093/brain/awh410.
23. **Horvath R, Hudson G, Ferrari G, Futterer N, Ahola S, Lamantea E, Prokisch H, Lochmuller H, McFarland R, Ramesh V, Klopstock T, Freisinger P, Salvi F, Mayr JA, Santer R, Tesarova M, Zeman J, Udd B, Taylor RW, Turnbull D, Hanna M, Fialho D, Suomalainen A, Zeviani M, Chinnery PF.** 2006. Phenotypic spectrum associated with mutations of the mitochondrial polymerase gamma gene. Brain **129:**1674–1684. http://dx.doi.org/10.1093/brain/awl088.